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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.(54) Title: **VECTORS FOR ENHANCED EXPRESSION OF VEGF FOR DISEASE TREATMENT**

(57) Abstract: The invention provides a vector which is capable of the expression of a vascular endothelial growth factor wherein said vector comprises a modified pCMV promoter. The invention further provides use of a vector which is capable of and expressing a vascular endothelial growth factor (VEGF) for the regulation of endothelial function, angiogenesis and arteriogenesis. The invention further comprises use of a vector which is capable of the expression of a vascular endothelial growth factor (VEGF) for the prophylactic treatment of arterial diseases and/or bone marrow diseases and/or neural diseases.

WO 02/095038 A2

Title: Vectors for enhanced expression of VEGF for disease treatment

The invention relates to the field of medicine. In particular it relates to the treatment of disease more specifically arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders.

In mammalian vascular development mesoderm-derived cells differentiate into endothelial cells that coalesce into blood vessels in a process called vasculogenesis. The formation of new blood vessels from pre-existing endothelium is termed angiogenesis. Several markers are consistently associated with embryonic stem (ES) cell-derived precursor cells having vascular potential, including the vascular endothelial growth factor (VEGF) receptor flk-1, the cell adhesion receptor platelet endothelial cell adhesion molecule (PECAM), and the adhesion molecule VE-cadherin.

VEGF-A functions in the development of embryonic structures during tissue remodelling and for the growth of tumour-induced vasculature. In solid tumors there is a constant requirement for vascular supply. Tumor-associated neovascularization is important for tumor cells to express their critical growth advantage. Experimental and clinical evidence suggests that the process of metastasis, is angiogenesis-dependent. Angiogenesis is a crucial process for tumor growth, metastasis and inflammation. Various angiogenic growth factors and cytokines induce neovascularization in tumors, namely members of the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) gene families. Vascular endothelial growth factor (VEGF) is thought to be the most potent angiogenic factor in numerous malignant tumors and is a prognostic indicator for cancer patients. A strong correlation has been found between VEGF expression and increased tumor microvasculature, malignancy, and metastasis, for example in breast cancer. Vascular endothelial growth factor (VEGF) signaling is required for both differentiation and proliferation of vascular endothelium. VEGF-A stimulates many actions of endothelial cells including proliferation, migration,

and nitric oxide release *via* binding to and activation of the two primarily endothelial-specific receptor-tyrosine kinases KDR and Flt-1. KDR and Flt-1 stimulate multiple signal transduction pathways in endothelial cells. These molecules also have in vivo expression patterns that are consistent with their being early markers of vascular lineage. VEGF signaling is critical for blood vessel formation during development.

In mouse VEGF is a 45-kd homodimer produced at sites of vasculogenesis and angiogenesis, and alternative splicing results in 3 different isoforms. The homodimer of VEGF-165 is the most active form and its properties include mitogenesis, chemotaxis, and permeability for endothelial cells. VEGF binds to 2 high-affinity receptors, flk-1 (VEGFR-2) and flt-1 (VEGFR-1), that are expressed in endothelium. Recently, a third molecule that binds VEGF with high affinity was identified as neuropilin-1, a receptor that also signals in the nervous system through a different ligand. Available data suggest that neuropilin-1 acts as a coreceptor with flk-1 in vascular tissues. Expression patterns of receptors and ligands suggest that VEGF may be a highly specific mediator of blood vessel formation *in vivo*, and analysis of targeted mutations in the mouse supports this hypothesis. Both flk-1 and flt-1 receptor mutations are recessive embryonic lethals at days 8.5 to 9.5 of gestation. The flk-1 mutation severely impairs vasculogenesis and hematopoiesis, whereas the flt-1 mutation affects vascular organization. Recent findings suggest that VEGF signaling is required for the transition of flk-1+ and PECAM+ cells to vascular endothelial cells that express ICAM-2 and CD34.

Dominantly acting transforming oncogenes are generally considered to contribute to tumor development and progression by their direct effects on tumor cell proliferation and differentiation. The growth of solid tumors beyond 1-2 mm in diameter requires the induction and maintenance of a tumor blood vessel supply, which is attributed in large part to the production of angiogenesis promoting growth factors by tumor cells. The mechanisms which govern the expression of angiogenesis growth factors in tumor cells are largely unknown, but

dominantly acting oncogenes are thought to have a much greater impact than hitherto realised. An example of this is the induction of expression of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) by mutant H- or K-ras oncogenes, as well as v-src and v-raf, in transformed fibroblasts or epithelial cells. Tumor-derived vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) plays an important role in neovascularization and the development of tumor stroma. Besides VEGF/VPF, mutant ras genes are known to up-regulate the expression of a variety of other growth factors thought to have direct or indirect stimulating effects on angiogenesis, e.g. TGF-beta and TGF-alpha. This effect may be mediated through the ras-raf-MAP kinase signal transduction pathway, resulting in activation of transcription factors such as AP1, which can then bind to relevant sites in the promoter regions of genes encoding angiogenesis growth factors. In principle, similar events could take place after activation or over-expression of many other oncogenes, especially those which can mediate their function through ras-dependent signal transduction pathways.

Excess blood vessel formation contribute to initiating and maintaining many diseases such as chronic inflammatory disorders, tumor growth, restenosis, and atherosclerosis. In contrast insufficient blood vessel formation is responsible for tissue ischemia, as in coronary artery disease. The treatment of vascular disease although greatly improved over recent decades by drug medication, surgical and minimally-invasive techniques, remains limited by vascular proliferative lesions and by our inability to modulate the progression of native disease. An increasing number of patients with advanced coronary artery disease remain symptomatic despite maximal interventional, surgical or medical treatment. Ideally, they would benefit most from additional arterial blood supply to ischemic areas of myocardium. The invention discloses a novel therapeutic strategy for the treatment of vascular disease using the angiogenic growth factor VEGF. Previous therapeutic strategies using VEGF comprising proteins and/or plasmids were not

able to produce VEGF polypeptide in a mammalian cells in sufficient quantities to improve neovascularisation or regional myocardial blood flow.

The invention provides a vector which is capable of effecting the expression of a vascular endothelial growth factor (VEGF or a functional equivalent thereof) in the appropriate environment wherein said vector comprises a modified pCMV promoter. The definition "functional equivalent" (homologue) means that a particular subject sequence varies from the reference sequence by one or more substitutions, deletions, or additions resulting in a sequence that encodes the same activity as VEGF in kind, not necessarily in amount. A vector capable of the effecting the expression of VEGF as used herein is a vector comprising a VEGF nucleic acid, that is at least capable of being expressed in the appropriate environment (i.e. a vector that is at least operative in mammalian systems). Preferably said vector comprises a plasmid. Preferably said modified pCMV promoter comprises the insertion of a 9bp nucleic acid sequence ACGCGCGCT, wherein A is a deoxyadenyl, G is deoxyguanyl, C is deoxycytosyl and T is thymidyl. pCMV promoter refers to the human cytomegalovirus (CMV) promoter. It is understood that (single) base substitutions may be made in said promoter sequence that will not significantly affect the function of the sequence.

The invention also provides a vector capable of effecting expression of VEGF, which lacks CpG-rich regions. Prior art vectors often had ampicillin resistance sequences or other sequences promoting immunogenicity such as CpG-islands. This may have contributed to the lack of efficacy in the prior art.

The invention provides a vector capable of effecting the expression of a vascular endothelial growth factor (VEGF) wherein said vector comprises an antibiotic resistance gene. Preferably said antibiotic resistance gene comprises kanamycin. Even more preferred said vector comprises a 5' HSV translation initiation signal and/or a β -globin splicing/adenylation signal. Also considered are all viral and eukaryotic, preferably mammalian, translation initiation signals and splicing/adenylation signals. Exogenous transcriptional elements and

initiation codons can be used and also can be of various origins, both natural and synthetic. The invention further provides a vector which is capable of effecting the expression of a vascular endothelial growth factor (VEGF), wherein said vector comprises a ColE1-like replicon and/or an F1 replication origin. ColE1-like replicons (e.g., pBR322) and other ColE1-like replicons (pMB1-, p15A, RSF1030-, and CloDF13-derived) in *E. coli* are considered suitable. F1 replication origin refers to phage F1 origin. All known replication origins previously defined and yet to be defined are deemed suitable. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for and least operative in mammalian systems.

In a preferred embodiment the invention provides a vector capable of effecting the expression of a vascular endothelial growth factor (VEGF), wherein said vascular endothelial growth factor (VEGF) is derived from a mammal. Preferably said mammal is a human. The invention further provides a vector capable of effecting the expression of a vascular endothelial growth factor (VEGF), wherein said vector comprises a vascular endothelial growth factor (VEGF) nucleic acid. Nucleic acid as used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represents the sense or antisense strand. It is understood that it is advantageous to increase the production of the endogenous vascular endothelial growth factor (VEGF) in a mammalian system, to promote angiogenesis, for example for the prophylactic treatment of arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders, in particular tissue ischemia especially associated with diabetes. In a preferred embodiment said nucleic acid is operative in mammalian systems.

In the case of mammalian expression vectors, the expression of a nucleic acid of the invention may be driven by a number of previously defined and yet to be defined promoters, including inducible, developmentally regulated and tissue specific promoters. Promoters or enhancers derived from the genomes of

mammalian cells are considered suitable. The invention further provides the use of the individual promoter of the nucleic acid of the present invention for this purpose. In particular any promoters of nucleic acids particularly involved in the regulation of endothelial function, angiogenesis or arteriogenesis are deemed suitable.

The invention provides a vector according to the invention wherein said vector can replicate to a high copy number in a bacterial cell. The invention further provides a host cell which comprises a vector according to the invention. The definition host cell as used herein refers to a cell in which an foreign process is executed by bio-interaction, irrespective of the cell belonging to a unicellular, multi-cellular, a differentiated organism or to an artificial cell or cell culture. Preferably said host cell is a mammalian cell, more preferred a human cell. The invention provides a host cell which contains within its genome a recombinant nucleic acid according the invention.

In a preferred embodiment the invention provides use of a vector which is suitable and capable of effecting the expression of a vascular endothelial growth factor (VEGF) according to the invention for the regulation of endothelial function. Vascular endothelial growth factor (VEGF) is also a specific endothelial cell mitogen that stimulates endothelial function and angiogenesis and plays a crucial role in tumor growth/angiogenesis. VEGF has a variety of effects on vascular endothelium, including the ability to promote endothelial cell viability, mitogenesis, chemotaxis, and vascular permeability. Vascular endothelial growth factor-A (VEGF-A) acts on endothelial cells and monocytes, two cell types that participate in the angiogenic and arteriogenic process in vivo. Monocytes are important in arteriogenesis and that their ability to migrate may be critical to the arteriogenic response. VEGF-A stimulates monocyte migration in healthy individuals.

The invention further provides use of a vector which is suitable and capable of effecting the expression of a vascular endothelial growth factor (VEGF) according to the invention for the regulation of angiogenesis. VEGF signaling is

critical for blood vessel formation during development. VEGF plays an important role in the development of the vascular system, wound healing, vascularization of tumors, and for angiogenesis in ischemic tissues including the heart. The invention provides for use of a vector according to the invention for promoting neovascularization of ischemic tissues, as a preventative treatment or early stage treatment of cardiovascular diseases. VEGF-A can initiate the process of vascularization by stimulating chemoattraction and proliferation of angioblasts and endothelial cells [i.e. VEGF-A expression can stimulate angiogenic remodeling]. VEGF is also a potent vasodilating agent. The invention provides for use of a vector according to the invention for promoting neovascularization of wounds. VEGF expression is involved not only the formation of a vascular network but also promotes tissue formation. VEGF is also required for the cyclical blood vessel proliferation in the female reproductive tract.

The invention further comprises the use of a vector which is suitable and capable of effecting the expression of a vascular endothelial growth factor (VEGF) according to the invention for the regulation of arteriogenesis. In a preferred embodiment the invention provides the use of a vector according to the invention for the regulation of coronary and peripheral artery angiogenesis. VEGF-A plays a role as an endogenous activator of coronary collateral formation in the human heart. In a preferred embodiment said vector capable of effecting the expression of VEGF according to the invention can be used in the prophylactic treatment of myocardial tissue (diseases), and also in the reconstructing process of infarcted myocardial tissue.

The invention further provides use of a vector according to the invention to induce the release of hematopoietic growth factors by bone marrow endothelial cells. VEGF is also required for longitudinal bone growth and endochondral bone formation. VEGF can induce the release of hematopoietic growth factors (GM-CSF) by bone marrow endothelial cells. In a preferred embodiment said vector capable of effecting the expression of VEGF according to the invention can be used to induce the release of hematopoietic growth factors, for example GM-CSF,

to accelerate hematologic recovery after bone marrow grafting. Hematopoietic growth factors can mobilize peripheral blood stem cells from the bone marrow to therapeutically intervene in accelerating hematologic recovery. In a preferred embodiment said vector capable of effecting the expression of VEGF according to the invention may be used for the prophylactic treatment of haematological and oncological diseases. Preferably said vector capable of effecting the expression of VEGF according to the invention may be used to release hematopoietic growth factors in tissues after myeloablative chemotherapy and hematopoietic stem cell grafting. This can reduce the mortality related to autologous and allogeneic graft failure (i.e. bone marrow grafting). The invention further provides a vector capable of effecting the expression of VEGF according to the invention may be used to release hematopoietic growth factors which may be used to increase the quality of cytopheresis peripheral stem cell harvesting.

The invention further provides use of a vector capable of effecting the expression of VEGF according to the invention to induce transendothelial progenitor cell migration. Preferably to induce stromal cell-derived factor-1 (SDF-1) driven transendothelial progenitor cell migration. In the presence of VEGF in-vitro stromal cell-derived factor-1 (SDF-1) can drive and thus increase transendothelial progenitor cell migration. This may be due to pore formation (increased endothelial fenestration). Transendothelial migration of progenitors in vitro is substantially enhanced by the chemokine stromal-cell-derived factor-1 (SDF-1), which is produced by bone marrow stromal cells. More primitive progenitors also respond to this chemokine. Transendothelial progenitor cell migration is regulated by adhesion molecules, paracrine cytokines, and chemokines. Mobilizing hematopoietic growth factors stimulate proliferation of hematopoietic cells, which may indirectly result in changes of the local cytokine and chemokine milieu, adhesion molecule expression, and eventually the mobilization of hematopoietic progenitor cells.

The invention provides use of a vector capable of effecting the expression of VEGF according to the invention to increase endothelial fenestration. In tissues

outside the brain, vascular endothelial growth factor-A (VEGF) causes vascular hyper-permeability by opening of inter-endothelial junctions and induction of fenestrations and vesiculo-vacuolar organelles (VVOs).

The invention provides use of a vector capable of effecting the expression of VEGF according to the invention to modulate neuroblastoma cell growth. VEGF may affect neuroblastoma cell growth directly and could be an autocrine growth factor. In a preferred embodiment said vector capable of effecting the expression of VEGF according to the invention can be used in the prophylactic treatment of neural diseases, and in the reconstruction process after neural injury.

Critical limb ischemia and diabetes mellitus

Critical limb ischemia is one of the most burdensome problems in diabetes treatment, and provides a major challenge for VEGF gene transfer:

- Patients with diabetes mellitus have a 20-fold risk of developing clinical peripheral arterial disease, a 17-fold increased risk of developing foot gangrene, and a 15-fold increased risk of amputation. In the Netherlands in 1992, 1810 amputations were performed. Actually 50% of lower leg amputations in non-trauma patients is performed in diabetes mellitus. Patients with diabetes mellitus have after unilateral amputation a 40-55% risk of amputation of the contralateral limb in the next 5 years. While the mean admission duration for clinical cases with a primary diagnosis of peripheral arterial disease is 12.2 days, this increases to 30 days in diabetes mellitus. Thus, in diabetes mellitus the prevalence of CLI is not only increased, but its course is markedly more serious.
- The localization of macrovascular lesions (stenosis/occlusions) in diabetes mellitus shows a predilection for distal vessels. Especially involvement of vessels below the knee is prominent. On top of this, microvascular involvement is specific for diabetes mellitus. The localization of these lesions makes them less accessible to conventional forms of revascularisation (surgery, PTA, stent).

- The current range of treatment options in CLI in diabetic foot disease is quite small. If possible revascularisation procedures are performed, and in case of superimposed infections aggressive antibiotic treatment is given. However, medical treatment is limited to prostacyclin analogues and attempts to improve tissue oxygenation with rheological means (e.g. Isodex), both with limited success. Initial results from a clinical study show less amputations and better limb survival of as a result of the VEGF treatment for the mentioned disease. Thus gene transfer of a VEGF vector according to the invention is useful for the treatment of tissue ischemia.

The invention further provides use of a vector capable of effecting the expression of VEGF according to the invention in the preparation of a composition. Suitable base for compositions are known in the art. The invention further provides a composition comprising a vector capable of effecting the expression of VEGF according to the invention. Preferably said composition is in a form that can be administered to a mammal. A preferred embodiment is that said composition is suitable for human external application, for example wound healing.

The invention also provides a composition comprising a vector capable of effecting the expression of VEGF according to the invention which is a pharmaceutical. Suitable pharmaceutical compositions are known and they may be in dosage forms such as tablets, pills, powders, suspensions, capsules, suppositories, injection preparations, ointments, eye drops etc.

In a preferred embodiment the invention provides use of a composition according to the invention and/or a vector according to the invention for the treatment of arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders, in particular tissue ischemia especially associated with diabetes. Modes of administration can readily be determined by conventional protocols. A preferable mode of administration is by syringe injection to a localized tissue.

The invention further comprises a method of treatment of arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders and/or wounds comprising administering a vector according to the invention and/or a composition according to the invention with a carrier to a suitable recipient. Preferably said carrier is a pharmaceutically acceptable carrier (e.g. drug carrier system) or inert carrier.

Examples

Example 1: Construction of a highly efficient VEGF expression plasmid (phVEGF165.MB) without an ampicilline resistant gene.

Plasmid phVEGF165.MB (figure 1) was constructed from phVEGF165.SR, which was originally constructed by cloning a VEGF cDNA, obtained by RT-PCR of human vascular smooth muscle cells, into a eukaryotic expression vector (see Severne et al., 1988 and Isner et al., 1996 for details). The plasmid phVEGF165.SR (kindly provided by Isner), was re-sequenced. Besides a few mutations (including in the AhdI site), an almost complete β -lactamase gene was found. The 3' part of the ampicillin resistance gene (751 bp) was deleted out of phVEGF165.SR.

Deleting of the 3' part of β -lactamase gene (751bp)/Creating AhdI site:

The PCR-forward primer: 5'-ATCGACATCCAGTCAAAGCCACGTTGTGTCTCA-3' (first 2 nucleotides are miscellaneous, followed by an AhdI site), and the PCR reverse primer: 5'-ATAGCATGCGAGTTTTTCGCCCCGAAGA-3', were used to amplify kanamycin.

For ColE1-origin the forward primer: 5'-

GCGGACTAGTGCTGTCCCTCTTCTCTTATGA-3' and the backward or reversed primer: 5'-GCCGACGTGCAGTCTAGGTGAAGATCCTTTT-3' (increased with AhdI) were used.

Both PCR products were ligated to each other, using AhdI, in an pCR-BluntII-TOPO (Invitrogen). And after sequencing, the kanamycin/ori gene was digested with ClaI and BlnI, and ligated into the ClaI/BlnI sites of phVEGF165.SR. This resulted in a plasmid without ampicillin and with a new AhdI restriction site: phVEGF165.MB. The promotor region was changed during growth in the E. Coli DH5-alpha and included in this promoter was a 9bp nucleic acid sequence

ACGCGCGCT insertion. Synthesis of VEGF mRNA is driven by the modified cytomegalovirus (CMV) promoter. Downstream of the VEGF coding region is a rabbit β -globin sequence for splicing and polyadenylation. A 5' untranslated thymidine kinase sequence from herpes simplex virus (HSV) is used for translation initiation. The phVEGF165.MB plasmid harbours the kanamycin gene for selection during propagation in *E.Coli*.

Positions in the plasmid:

| | | |
|-----------|---|----------|
| 14- 619 | CMV promoter/enhancer | - CMV |
| 620- 682 | 5'HSV translation initiation signal | - 5'HSV |
| 682-1258 | VEGF coding sequence | - VEGF |
| 1259-2180 | β -globin splicing/polyadenylation signal | - pA |
| 2457-3370 | ColE1-like replicon | - ori |
| 3388-4623 | kanamycin gene | - kana |
| 4647-4724 | 5' part of β -lactamase gene | |
| 5238-5690 | f1 origin | - f1 ori |
| 5854-5875 | T7 promoter | - T7 |

Example 2. Detection of the ability of the phVEGF165MB plasmid to transform COS7 cells to produce VEGF protein.

70% confluent COS7 cells were transfected with VEGF plasmid Fugene. After 3 and 4 days the VEGF concentration in the supernatant was detected with a VEGF R&D kit (ELISA). The VEGF production (n=3) on day 3 and 4 of the plasmids without (phVEGF165.RS) and with (phVEGF165.MB) the modified cytomegalovirus (CMV) promoter were respectively 1.3/1.5 and 1.4/1.6 (pg/ml per 10E6 COS cells).

Phase I clinical studies have established that intramuscular gene transfer may be utilized to successfully accomplish therapeutic angiogenesis.

Gene transfer was performed in ten limbs of nine patients with non-healing ischemic ulcers (n=7) and/or rest pain (n=10) due to peripheral arterial disease. A total amount of 4000 µg naked plasmid DNA encoding the secreted 165-amino acid isoform of human VEGF (phVEGF₁₆₅) was injected into ischemic muscles of the affected limb. The average follow-up was 6 ± 3 (range 2 to 11) months. Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial CPK measurements remained in the normal range and there were no signs of systemic or local inflammatory reactions. To date, no aggravated deterioration in eyesight due to diabetic retinopathy or growth of latent neoplasm has been observed in any patient treated with phVEGF₁₆₅ gene transfer. The only complication observed in the trial, was limited to transient lower extremity edema, consistent with VEGF-enhancement of vascular permeability.

Transgene expression. Blood levels of VEGF transiently peaked one to three weeks post gene transfer in seven patients amenable for weekly assays. In two patients baseline and/or more than two follow-up blood samples were not achievable. Clinical evidence of VEGF (vascular permeability factor) overexpression was evident by the observation of peripheral edema development (+1 to +4 by gross inspection) in those six patients with ischemic ulcers. In four patients, the edema was limited to the treated limb, while in two patients the contralateral limb was affected as well, albeit less severely. Edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive arterial testing. The absolute systolic ankle or toe pressure increased in nine limbs post gene transfer and was unchanged in one limb at the time of the most recent follow-up ($p=0.008$). The ABI and/or TBI increased from 0.33 ± 0.04 (0.22 to 0.57, $p=0.028$, [n=10]) at four weeks; to 0.45 ± 0.04 (0.27 to 0.59, $p=0.016$, [n=10]) at eight weeks; and to 0.48 ± 0.03 (0.27 to 0.67, $p=0.017$, [n=8])

at 12 weeks. Improvement in the pressure index was sustained, but did not further rise significantly after the second gene transfer.

Exercise performance improved in all five patients with rest pain or minor ischemic ulcers, who underwent a graded treadmill exercise. All patients experienced a significant increase in pain-free walking time (2.5 ± 1.1 min pre gene therapy vs 3.8 ± 1.5 min at an average of 13 weeks post gene therapy, $p=0.043$) and absolute, claudication-limited walking time (4.2 ± 2.1 min vs 6.7 ± 2.9 min, $p=0.018$). Two patients reached the target endpoint of ten minutes of exercise.

Angiography. Digital subtraction angiography showed newly visible collateral vessels at the knee, calf and ankle levels in six of ten ischemic limbs treated. The luminal diameter of the newly visible vessels ranged from 200 μ m to > 800 μ m, although most were closer to 200 μ m and these frequently appeared as a "blush" of innumerable collaterals. Collaterals did not regress in follow-up angiograms. Magnetic resonance angiography showed qualitative evidence of improved distal flow with enhancement of signal intensity as well as an increase in the number of newly visible collaterals in eight limbs.

Change in limb status and ischemic rest pain. Therapeutic benefit was demonstration by regression of rest pain and/or improved limb integrity. The frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly (5.9 ± 2.1 at baseline vs 1.5 ± 2.8 at eight week follow-up, $p=0.043$), with a slight reduction of analgesic medication (on average from 1.8 to 1.5 analgetics/24h period). Based on criteria proposed by Rutherford, limb status improved in nine of ten extremities treated. Moderate improvement, including both an upward shift in the clinical category (at least one clinical category in patients with rest pain and at least two categories to reach the level of claudication in patients with tissue loss) and an increase in the ABI > 0.1 was documented in five cases. In one patient an ischemic ulcer resolved sufficiently to

permit placement of a split-thickness skin grafting, leading to absolute limb salvage. In two patients, in whom a major amputation would have been inevitable, retention of a functional foot by a minor (toe) amputation was reached. Minimal improvement, including an upward shift in clinical category or improvement of the ABI > 0.1 was present in another three cases. However, in two patients with and extensive forefoot necrosis and osteomyelitis a below-knee amputation was required despite significant hemodynamic and angiographic improvement. There was one patient with progressive toe gangrene, who remained unchanged from his hemodynamic and angiographic findings. The patient underwent a below-knee amputation eight weeks after gene therapy.

Immunohistochemistry and molecular analysis. Tissue specimens derived from one amputee ten weeks after gene therapy showed foci of proliferating endothelial cells. This finding was particularly striking with the fact in mind, that endothelial cell proliferation is nearly absent in normal arteries, is consistent with an estimated endothelial cell turnover time of "thousands of days" is quiescent microvasculature. PCR performed on these samples indicated persistence and widespread distribution of DNA fragments unique to phVEGF₁₆₅. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the site of injection. Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimen derived from two amputees eight and ten weeks after gene therapy.

Example 3. A further study was performed to determine the effect of treating diabetic patients with critical limb ischemia with VEGF.

Ischemic muscle represents a promising target for gene therapy with naked plasmid DNA. Intramuscular (IM) transfection of genes encoding angiogenic cytokines, particularly those which are naturally secreted by intact cells, may

constitute an alternative treatment strategy for patients with extensive tissue ischemia, in whom contemporary therapies (pharmacologic interventions, angioplasty, bypass surgery) have previously failed or are not feasible. This strategy is designed to promote the development of supplemental collateral blood vessels that will constitute endogenous bypass conduits around occluded native arteries, a strategy termed "therapeutic angiogenesis".

Pre-clinical animal studies have indeed indicated that IM gene transfer may be utilized to successfully accomplish therapeutic angiogenesis. More recently, phase I clinical studies have indicated that IM gene transfer may be utilized to safely and successfully accomplish therapeutic angiogenesis in patients with critical limb ischemia.

At this moment there is no standard pharmacotherapeutic treatment.

Antithrombotic and vasoactive drugs are of little value in the management of CLI. Only prostanoids have shown some efficacy, but there is no evidence that in the long-term the limb is saved.

The protocol outlined has been designed as a double blind placebo controlled phase III study of direct intramuscular gene transfer of phVEGF₁₆₅ in patients with critical limb ischemia (CLI).

The objectives are to determine the clinical response and physiologic extent of collateral artery development in patients receiving intramuscular phVEGF₁₆₅ gene transfer and the safety of this treatment on diabetic retinopathy and nephropathy compared with placebo. The primary endpoints are limb survival 100 days after the first IM injection with VEGF and an increase of ABI of 15%. A total of 60 adult men and women will participate in this study. Subjects will be eligible if they have critical limb ischemia, diabetes and not to be optimal candidates for surgical or percutaneous revascularization. The clinical response of subjects treated in this fashion will be evaluated by serial studies performed

before and after treatment, non invasive perfusion techniques, capillary microscopy and PET.

PROTOCOL

I. Therapeutic angiogenesis is a novel strategy for the treatment of ischemia.

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago (1). Their work documented the extent to which tumor development was dependent upon neovascularization and suggested that this relationship might involve angiogenic growth factors which were specific for neoplasms. Beginning a little over a decade ago (2), a series of polypeptide growth factors were purified, and demonstrated to be responsible for natural as well as pathologic angiogenesis.

Subsequent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. This novel strategy for the treatment of vascular insufficiency has been termed "therapeutic angiogenesis" (3). The angiogenic growth factors first employed for this purpose comprised members of the FGF family. Baffour et al administered bFGF in daily intramuscular doses of 1 or 3 µg to rabbits with acute hindlimb ischemia; at the completion of 14 days of treatment, angiography and necropsy measurement of capillary density showed evidence of augmented collateral vessels in the lower limb, compared to controls (4). Pu et al used an acidic fibroblast growth factor (aFGF) to treat rabbits in which the acute effects of surgically-induced hindlimb ischemia were allowed to subside for 10 days before beginning a 10-day course of daily 4 mg IM injections; at the completion of 30 days follow-up, both angiographic and hemodynamic evidence of collateral development was superior to ischemic controls treated with IM saline (5). Yanagisawa-Miwa et al likewise demonstrated the feasibility of bFGF for salvage of infarcted myocardium, but in this case growth factor was administered intra-

arterially at the time of coronary occlusion, followed 6 hrs later by a second intra-arterial bolus (6).

Isner used the same animal model developed by Pu et al (5) to investigate the therapeutic potential of a 45 kDa dimeric glycoprotein, vascular endothelial growth factor (VEGF), isolated initially as a heparin-binding factor secreted from bovine pituitary folliculo-stellate cells (7). VEGF was also purified independently as a tumor-secreted factor that induced vascular permeability by the Miles assay (8, 9), and thus its alternate designation, vascular permeability factor (VPF). Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH₂ terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells (10). Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors *Flt-1* (11) and *Flk-1* / KDR (12, 13) are present on endothelial cells, but not other cell types; consequently, the mitogenic effects of VEGF - in contrast to acidic and basic FGF, both of which are known to be mitogenic for smooth muscle cells (14, 15) and fibroblasts as well as endothelial cells - are limited to endothelial cells (7, 16). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis) (17, 18). Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (19, 20), the chorioallantoic membrane (7), and the rabbit bone graft model (20). The hypothesis was that the angiogenic potential of VEGF was sufficient to constitute a therapeutic effect (21). This hypothesis was confirmed after the administration of soluble 165-amino acid isoform of VEGF (VEGF₁₆₅) as a single intra-arterial bolus to the internal iliac artery of rabbits in which the ipsilateral femoral artery was excised to induce severe, unilateral hindlimb ischemia. Doses of 500-1.000 µg of VEGF produced statistically significant augmentation of angiographically visible collateral vessels, and histologically identifiable capillaries; consequent amelioration of the hemodynamic deficit in the ischemic limb was significantly greater in animals receiving VEGF than in non-treated controls (calf blood

pressure ratio = 0.75 ± 0.14 vs 0.48 ± 0.19 , $p < 0.05$). Serial (baseline, as well as 10 and 30 days post-VEGF) angiograms disclosed progressive linear extension of the collateral artery of origin (stem artery) to the distal point of parent-vessel (reentry artery) reconstitution in 7 of 9 VEGF-treated animals. Similar results were achieved in a separate series of experiments in which VEGF was administered by an intramuscular route daily for 10 days (22). These findings thus established proof of principle for the concept that the angiogene activity of VEGF is sufficiently potent to achieve therapeutic benefit.

While each of these studies documented an increase in the number of angiographically visible collaterals, and increased capillary density in the muscles studied at necropsy, evidence regarding the physiological consequences of such anatomical improvement was limited to blood pressure measurements recorded in the ischemic versus the normal limb. Accordingly, a series of studies in the ischemic hindlimb model in which an intra-arterial Doppler wire (23), sufficiently diminutive (0.018 in.) to measure phasic blood flow velocity in the rabbit's internal iliac artery, was used to investigate resting and maximum flow following therapeutic angiogenesis with a single, intra-arterial bolus of VEGF₁₆₅. By 30 days post-VEGF₁₆₅, flow at rest, as well as maximum flow velocity and maximum blood flow provoked by 2 mg papaverine were all significantly higher in the VEGF-treated group (24).

One of the distinguishing features of VEGF mentioned above - the fact that the VEGF gene encodes a secretory signal sequence - might be exploited as part of a strategy designed to accomplish therapeutic angiogenesis by arterial gene transfer. Previously was observed that site-specific transfection of rabbit ear arteries with the plasmid pXGH5 encoding the gene for human growth hormone - a secreted protein - yields local levels of human growth hormone equivalent to what has been considered to be in a physiologic range, despite the fact that immunohistochemical examination of the transfected tissue disclosed evidence of successful transfection in $< 1\%$ of cells in the transfected arterial segment (25). Thus, gene products which are secreted may have profound biological effects,

even when the number of transduced cells remains low. In contrast, for genes such as bFGF which do not encode a secretory signal sequence, transfection of a much larger cell population might be required for that intracellular gene product to express its biological effects.

Therefore, 400 µg of phVEGF₁₆₅ was applied, encoding the 165-amino acid isoform of VEGF, to the hydrogel layer coating the outside of an angioplasty balloon (26) and delivered the balloon catheter percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause hindlimb ischemia. Site-specific transfection of phVEGF₁₆₅ was confirmed by analysis of the transfected internal iliac arteries using reverse transcriptase-polymerase chain reaction (RT-PCR) and then sequencing the RT-PCR product. Augmented development of collateral vessels was documented by serial angiograms in vivo, and increased capillary density at necropsy. Consequent amelioration of the hemodynamic deficit in the ischemic limb was documented by improvement in the calf blood pressure ratio (ischemic/normal limb) to 0.70 ± 0.08 in the VEGF-transfected group vs 0.50 ± 0.18 in controls ($p < 0.05$). Angiographic and histologic evidence of angiogenesis were subsequently demonstrated following intra-arterial gene transfer of phVEGF₁₆₅ in a human patient (27). These findings established that site-specific gene *transfer* can be used to achieve physiologically meaningful *therapeutic* modulation of vascular disorders, including therapeutic angiogenesis. Of note, no study have disclosed any evidence of immunologic toxicity.

II. Critical limb ischemia and diabetes mellitus

Critical limb ischemia is one of the most burdensome problems in diabetes treatment, and provides a major challenge for VEGF gene transfer:

- Patients with diabetes mellitus have a 20-fold risk of developing clinical peripheral arterial disease, a 17-fold increased risk of developing foot gangrene, and a 15-fold increased risk of amputation. In the Netherlands in 1992 1810 amputations were performed. Actually 50% of lower leg amputations in non-

trauma patients is performed in diabetes mellitus. Patients with diabetes mellitus have after unilateral amputation a 40-55% risk of amputation of the contralateral limb in the next 5 years. While the mean admission duration for clinical cases with a primary diagnosis of peripheral arterial disease is 12.2 days, this increases to 30 days in diabetes mellitus. Thus, in diabetes mellitus the prevalence of CLI is not only increased, but its course is markedly more serious.

- The localization of macrovascular lesions (stenosis/occlusions) in diabetes mellitus shows a predilection for distal vessels. Especially involvement of vessels below the knee is prominent. On top of this, microvascular involvement is specific for diabetes mellitus. The localization of these lesions makes them less accessible to conventional forms of revascularisation (surgery, PTA, stent).

- The current range of treatment options in CLI in diabetic foot disease is quite small. If possible revascularisation procedures are performed, and in case of superimposed infections aggressive antibiotic treatment is given. However, medical treatment is limited to prostacyclin analogues and attempts to improve tissue oxygenation with rheological means (e.g. Isodex), both with limited success.

- On the other hand, other diabetic complications may increase the risk of side effects of VEGF therapy in diabetes patients. Especially proliferative retinopathy, but also less outspoken forms of retinopathy and microalbuminuria might exacerbate in case of systemic effects of VEGF. Even the nature of the local abnormalities in CLI in diabetes might compromise beneficial effects of VEGF: in diabetes mellitus total skin flow is not necessarily reduced, but the contribution of nutritive capillary versus shunt skin flow may be lowered. It is at this stage not known what effect intramuscular administration of VEGF may have on this distribution of skin flow, and, clinically, on ulcer healing.

In conclusion, CLI in patients with diabetes there is no standard treatment, VEGF may increase both the chance of beneficial and unwanted effects of VEGF in patients with diabetes mellitus (41,42,43).

III. Gene transfer of cDNA encoding for secreted protein.

In experiments which have relied exclusively on the use of non-secreted gene products, examination by histochemical staining, in situ hybridization, and/or polymerase chain reaction has suggested that the transfection efficiency of direct gene transfer to vascular smooth muscle cells within the arterial wall was considerably less than 1% and might therefore preclude a meaningful biological response. In contrast, genes encoding for a secreted protein may overcome the handicap of inefficient transfection by a paracrine effect, secreting adequate protein to achieve local levels that may be physiologically meaningful. Nabel et al (28) demonstrated that despite similarly low efficiencies, cell surface protein expression resulting from percutaneous transfection of vascular smooth muscle cells with the histocompatibility gene HLA-B7 may be adequate to induce a biological response, namely, focal vasculitis. Necropsy evidence of a pathobiological response following arterial gene transfer was reported by the same group in the case of transgenes encoding for the secreted proteins PDGF-B (29) and FGF-1 (30); in the former study, only 0.1 to 1% of cells in the artery segment were estimated to contain plasmid DNA by PCR approximation. To more specifically determine the relation between a secreted gene product and transfection efficiency after in vivo arterial gene transfer, in vitro (31) and in vivo (25) models to serially monitor expression of a gene encoding for a secreted protein. In vivo analyses were performed using the central artery of the rabbit ear. Liposome-mediated transfection of plasmid DNA containing the gene for human growth hormone (hGH) was successfully performed in 18 of 23 arteries. Serum hGH levels measured 5 days after transfection ranged from 0.1 to 3.8 ng/mL (mean, 0.97 ng/mL); in contrast, serum drawn from the control arteries demonstrated no evidence of hGH production. Serial measurement of hGH from transfected arteries demonstrated maximum hGH secretion 5 days after transfection and no detectable hormone after 20 days. Despite these levels of secreted gene product documented in vivo, immunohistochemical staining of

sections taken from the rabbit ear artery at necropsy disclosed evidence of successful transfection in <0.1% of cells in the transfected segment. Thus, low-efficiency transfection with a gene encoding for a *secreted* protein may achieve therapeutic effects not realized by transfection with genes encoding for proteins which remain intracellular.

In conclusion - in combination with the fact that ischemic skeletal muscle itself serves to augment transfection efficiency (32, 33) - similarly accounts for the bioactivity that is described above of gene transfer of naked DNA by direct injection into skeletal muscle (*vide infra*).

IV. Pre-clinical animal studies.

Ten days after ischemia was induced in one hindlimb of New Zealand White rabbits, 500 µg of phVEGF₁₆₅, or the reporter gene LacZ, were injected IM into the ischemic hindlimb muscles. Site-specific transgene expression was documented by mRNA and immunohistochemistry. At 30-day follow-up, angiographically recognizable collateral vessels and histologically identifiable capillaries were increased in VEGF-transfectants compared to controls. This augmented vascularity improved perfusion to the ischemic limb, as documented by a superior calf blood pressure ratio for phVEGF₁₆₅ (0.84 ± 0.09) vs controls (0.67 ± 0.06 , $p < .01$); by improved blood flow in the ischemic limb (measured using an intra-arterial Doppler wire) at rest (phVEGF₁₆₅ = 52.5 ± 12.6 , control = 38.4 ± 4.3 , $p < .05$); and by increased distribution of labeled microspheres to the adductor muscle (phVEGF₁₆₅ = 4.3 ± 0.5 , control = 2.9 ± 0.6 ml/min/100g tissue, $p < .05$), as well as the gastrocnemius muscle (phVEGF₁₆₅ = 3.9 ± 0.8 , control = 2.8 ± 0.9 ml/min/100g tissue, $p < .05$) of the ischemic limb. A more detailed description of this study has been published previously (32).

Ischemic muscle thus represents a promising target for gene therapy with naked plasmid DNA. IM transfection of genes encoding angiogenic cytokines, particularly those which are naturally secreted by intact cells, may constitute an

alternative treatment strategy for patients with extensive tissue ischemia, in whom contemporary revascularization (anti-anginal medications, angioplasty, bypass surgery) have previously failed or are not feasible.

V. Phase I clinical studies.

Gene transfer was performed in ten limbs of nine patients with non-healing ischemic ulcers (n=7) and/or rest pain (n=10) due to peripheral arterial disease. A total amount of 4000 µg naked plasmid DNA encoding the secreted 165-amino acid isoform of human VEGF (phVEGF₁₆₅) was injected into ischemic muscles of the affected limb. The average follow-up was 6 ± 3 (range 2 to 11) months. Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial CPK measurements remained in the normal range and there were no signs of systemic or local inflammatory reactions. To date, no aggravated deterioration in eyesight due to diabetic retinopathy or growth of latent neoplasm has been observed in any patient treated with phVEGF₁₆₅ gene transfer. The only complication observed in the trial, was limited to transient lower extremity edema, consistent with VEGF-enhancement of vascular permeability (Baumgartner et al, Circ 1998; 97: 114-1123).

Transgene expression. Blood levels of VEGF transiently peaked one to three weeks post gene transfer in seven patients amenable for weekly assays. In two patients baseline and/or more than two follow-up blood samples were not achievable. Clinical evidence of VEGF (vascular permeability factor) overexpression was evident by the observation of peripheral edema development (+1 to +4 by gross inspection) in those six patients with ischemic ulcers. In four patients, the edema was limited to the treated limb, while in two patients the

contralateral limb was affected as well, albeit less severely. Edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive arterial testing. The absolute systolic ankle or toe pressure increased in nine limbs post gene transfer and was unchanged in one limb at the time of the most recent follow-up ($p=0.008$). The ABI and/or TBI increased from 0.33 ± 0.04 (0.22 to 0.57, $p=0.028$, [n=10]) at four weeks; to 0.45 ± 0.04 (0.27 to 0.59, $p=0.016$, [n=10]) at eight weeks; and to 0.48 ± 0.03 (0.27 to 0.67, $p=0.017$, [n=8]) at 12 weeks. Improvement in the pressure index was sustained, but did not further rise significantly after the second gene transfer.

Exercise performance improved in all five patients with rest pain or minor ischemic ulcers, who underwent a graded treadmill exercise. All patients experienced a significant increase in pain-free walking time (2.5 ± 1.1 min pre gene therapy vs 3.8 ± 1.5 min at an average of 13 weeks post gene therapy, $p=0.043$) and absolute, claudication-limited walking time (4.2 ± 2.1 min vs 6.7 ± 2.9 min, $p=0.018$). Two patients reached the target endpoint of ten minutes of exercise.

Angiography. Digital subtraction angiography showed newly visible collateral vessels at the knee, calf and ankle levels in six of ten ischemic limbs treated. The luminal diameter of the newly visible vessels ranged from 200 μm to $>800 \mu\text{m}$, although most were closer to 200 μm and these frequently appeared as a "blush" of innumerable collaterals. Collaterals did not regress in follow-up angiograms. Magnetic resonance angiography showed qualitative evidence of improved distal flow with enhancement of signal intensity as well as an increase in the number of newly visible collaterals in eight limbs.

Change in limb status and ischemic rest pain. Therapeutic benefit was demonstrated by regression of rest pain and/or improved limb integrity. The frequency of ischemic rest pain expressed as afflicted nights per week decreased

significantly (5.9 ± 2.1 at baseline vs 1.5 ± 2.8 at eight week follow-up, $p=0.043$), with a slight reduction of analgesic medication (on average from 1.8 to 1.5 analgetics/24 h period). Based on criteria proposed by Rutherford, limb status improved in nine of ten extremities treated. Moderate improvement, including *both* an upward shift in the clinical category (at least one clinical category in patients with rest pain and at least two categories to reach the level of claudication in patients with tissue loss) and an increase in the ABI >0.1 was documented in five cases. In one patient an ischemic ulcer resolved sufficiently to permit placement of a split-thickness skin grafting, leading to absolute limb salvage. In two patients, in whom a major amputation would have been inevitable, retention of a functional foot by a minor (toe) amputation was reached. Minimal improvement, including an upward shift in clinical category or improvement of the ABI >0.1 was present in another three cases. However, in two patients with an extensive forefoot necrosis and osteomyelitis a below-knee amputation was required despite significant hemodynamic and angiographic improvement. There was one patient with progressive toe gangrene, who remained unchanged from his hemodynamic and angiographic findings. The patient underwent a below-knee amputation eight weeks after gene therapy.

Immunohistochemistry and molecular analysis. Tissue specimens derived from one amputee ten weeks after gene therapy showed foci of proliferating endothelial cells. This finding was particularly striking with the fact in mind, that endothelial cell proliferation is nearly absent in normal arteries, is consistent with an estimated endothelial cell turnover time of "thousands of days" in quiescent microvasculature. PCR performed on these samples indicated persistence and widespread distribution of DNA fragments unique to phVEGF₁₆₅. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the site of injection. Southern blot analysis confirmed persistence of

intact plasmid DNA in muscle specimen derived from two amputees eight and ten weeks after gene therapy.

Experimental design

A. Objectives

The scope of this double blind phase III study is to compare the treatment of intramuscular (IM) gene transfer of phVEGF_{165s} in patients with critical limb ischemia with the standard treatment observation.

1. *Primary objectives*

To determine the effect of intramuscular administration of 4000 µg phVEGF_{165s} on clinical, physiological parameters of critical limb ischemia and safety compared to the standard treatment.

2. *Secondary objectives*

To determine and compare the effect on the quality of life for both treatments.

B. Selection of Patients

1. Inclusion Criteria

A total of 60 adult diabetic men and women will participate in this study. The restriction for diabetic patients provides a homogeneous population with similar vascular problems (peripheral vessels, frequently no alternative treatment, enough patients to participate). Subjects will be eligible if they have critical limb ischemia and have been judged not to be optimal candidates for surgical or percutaneous revascularization. Subjects must meet the following criteria to be eligible for study enrollment:

Male or female > 18 years of age.

Both type I and type II diabetes mellitus patients are candidates.

Presence of critical limb ischemia, according to the following definitions (European Consensus Group on Critical Limb Ischemia):

- rest pain and/or ischemic skin lesions, lasting for more than 2 weeks.
- ankle systolic blood pressure < 50 mmHg, or, in case of incompressible ankle vessels, toe systolic blood pressure < 30 mmHg (44).

Not optimal candidates for surgical or percutaneous revascularization as determined by angiography.

2. Exclusion Criteria

Subjects who meet any of the following criteria will be excluded from the study enrollment:

Acute surgery necessary.

Pregnancy, lactation, or use of inadequate contraception.

Evidence of cancer (except low grade and fully resolved non-melanoma skin malignancy)

Preproliferative or proliferative diabetic retinopathy, or conditions obscuring ophthalmological inspection of the retina (e.c. cataract), based on fluorescein angiography of the retina. Patients without retinopathy or with background retinopathy are allowed.

Serum creatinine > 200 μ M.

Concurrent participation in a study using an experimental drug or an experimental procedure within the 28 days before VEGF gene transfer.

Other severe concurrent illness (e.g., active infection, severe congestive heart failure, or left ventricular ejection fraction [EF] <20%).

Bleeding diathesis, HIV infection, or any other condition that, in the opinion of the investigator, could pose a significant hazard to the subject if the investigational therapy was to be initiated.

Inability to follow the protocol and comply with follow-up requirements.

C. Dose and Administration

1. Dosage

The clinical study is performed as a double blind phase III study in 60 patients. In half of the patients a total dose of 4000 ug ph VEGF₁₆₅ will be given, each patient will receive 4 injections (each with 500 µg phVEGF₁₆₅), each with a volume of 1 ml. This will be repeated after 4 weeks (day 28). The other half of the patients will receive injections with physiologic salt without phVEGF.

2. Administration

The VEGF gene via a small needle (27 G) will be injected directly into the muscle. With echography the position of the needle will be checked. The spreading of the injectate into the muscle will also be monitored in this way. After introduction of the needle 5 ml of saline or saline containing 500 µg phVEGF₁₆₅ will be injected. This will be done at four different injection sites in gastrocnemius and anticus muscles.

D. Concomitant therapy

No concomitant routine therapy will be excluded in this study. Subjects will be treated by their personal physicians with routine medication as needed. No restriction of medication will be stipulated. However, physicians will be encouraged not to change the established regimen of analgetics post intramuscular injections unless clearly indicated by a change in clinical status.

E. Pretreatment Assessments

Subjects will be screened prior to study initiation to determine their eligibility for the study based on the inclusion and exclusion criteria. Written informed consent will be obtained from subjects prior to intra muscular injections. To be eligible, the following assessments must be performed/obtained within 2 weeks prior to intra-muscular injection unless otherwise indicated:

Medical history, including medications, demographic information, vascular history, and history of cancer.

Review of systems and specific emphasis on symptoms or history suggestive of tumors and on reproductive status.

Pain assessment by the McGill Pain Questionnaire and the pain rating index (PRI).

Vital signs (blood pressure, pulse, respirations, temperature).

Physical examination:

- * Vascular status, using angiography and noninvasive vascular laboratory examinations, including ankle and toe pressures and Doppler spectral analysis or duplex examination of large lower extremity vessels. Criteria for CLI see above.

- * Detailed ophthalmological examination, including funduscopy, fundus photographs, and fluorescein angiography of retinal vessels.

- * Neurological examination, including quantified muscle force testing, neurographic examination of motor and sensory nerves of arm and leg, and quantified sensory examination.

Clinical laboratory samples:

- * SMAC-20 (sodium, potassium, bicarbonate, chloride, glucose, BUN, creatinine, albumin, alkaline phosphatase, bilirubin, calcium, creatine kinase, LDH, phosphate, SGOT [ALT] SGPT [AST], tropinins, CRP, total protein, uric acid, cholesterol, triglycerides; VEGF protein and plasmid; anti-dsDNA, two nightly portions of urine for micro-albuminuria).

- * Additional laboratory assessments: endothelial >markers= including: factor VIII-vWF, ICAM, VCAM, e-selectin.

- * CBC with manual differential and platelets.

- * Complete urinalysis, including dipstick for protein.

PT/aPTT or INR.

12-lead ECG.

Chest X-ray (posterior-anterior and lateral).

Serum pregnancy test for women of childbearing potential (within 24 hours prior to study drug administration).

F. Follow-Up Assessments

In hospital till three days after the injections: daily, vital signs, physical examination, use of analgetics, electrocardiogram, clinical laboratory testing (SMAC 20).

Follow-up clinic or office visit will be conducted when possible on an outpatient basis on Days 3, 7, 14 and 72 days after the intra muscular injections. The following assessments will be performed/obtained on Days 3, 7, 14 and 72 days unless otherwise indicated:

Vital signs (blood pressure, pulse, respirations, temperature).

Physical examination, including funduscopy.

Interval history (including medication changes) and review of systems.

Record the analgetics use in a diary.

Clinical laboratory samples SMAC-20 CBC with manual differential and platelets, urinalysis.

F1. Schedule of evaluation

[illegible]

| | | | | | | | | | | |
|--|---|--|--|--|---|---|---|---|---|---|
| technique s | X | | | | X | X | X | X | X | X |
| Angiogra phy | X | | | | | | | | | X |
| ophthalm ol- ogical examinat ion | X | | | | | X | | | | X |
| Neurologi c examinat ion | X | | | | | | | | | X |
| PET scan | X | | | | | | | | | X |

G. Analysis of clinical response

Patients will receive a diary to note their severity of pain (pain assessment by the McGill Pain Questionnaire and the PRI) and the daily use of analgetics. In the diary they also note their daily medication and activity.

H. Analysis of the quality of life

In this study the RAND-36 will be used to analyze the quality of life.

I. Analysis of physiological and anatomical activity

On the specific vascular methods, and the choice of primary and secondary measures of effect:

The clinical course of an episode of CLI may be quite variable. Although in some patients an inexorable downhill course to amputation follows, in others slow healing of ulcers may occur. Several risk factors for an adverse outcome have been identified, such as diabetes, continued smoking, concurrent other cardiovascular disease or vascular laboratory parameters like low ankle or toe pressure or TcPO₂. However, unpredictability of ulcer healing in an individual patient stresses the importance of a controlled design in an intervention study. This is also the major flaw in the clinical studies of VEGF gene transfer in CLI reported so far.

The increase in ankle pressures and ABI reported in the studies of Isner et al of VEGF gene therapy in CLI is remarkable. In fact non-revascularization interventions in CLI (medication, training) have never been reported to result in consistent increases in ankle pressures. In revascularisation studies a 15% increase in ankle pressures is considered to be clinically relevant (45, 46).

I.1. Available vascular techniques.

For the primary measure of effect:

I.1.1. Ankle- and thigh-brachial and toe-brachial pressure and index.

Using standard techniques and following AHA recommendations, the Doppler ankle blood pressure is measured and divided by a simultaneously measured brachial artery blood pressure (47). In short, using a 8 MHz Doppler probe forming part of a Parkes Doppler equipment audio Doppler signals are obtained over the posterior tibial and dorsalis pedis arteries of both feet. After suprasystolic inflation of previously applied cuffs adapted for ankle (12 cm wide) and thigh (18 cm wide) circumference, the ankle (and optionally: thigh) Doppler systolic blood pressure are measured during slow deflation. Measurements are made at rest. If the foot condition allows so, the measurement at rest will be followed by a measurement immediately after 3 minutes of exercise on a calf ergometer with a moment of 33 Nm. The toe pressure is determined using a toe cuff with photoplethysmographic assessment of the systolic blood pressure level during cuff deflation. Using an automated blood pressure meter simultaneous brachial artery blood pressures are obtained. The ankle-, thigh- and toe-brachial index is calculated by dividing the ankle blood pressure by the simultaneously measured brachial artery blood pressure. For the ankle blood pressure the highest of either posterior tibial or dorsalis pedis artery at rest is used, for the measurement after exercise the same foot artery is used. The CV of the measurements at rest has been found in several studies to be around 5% for intra-individual day-to-day comparisons (48,49,50). For inter-individual comparisons of technicians the CV amounts to 8% (48). For a post-exercise ankle-brachial index the CV amounts to 9% (51).

For secondary measures of effect:

I.1.2. Flow in calf/forearm using strain gauge plethysmography.

Calf blood flow. Resting and maximal calf blood flow can be measured non-invasively by plethysmography, using an ECG-triggered venous occlusion plethysmograph with a pneumatically powered cuff inflator allowing rapid flow measurements. Flow measurements at rest are made in subjects by alternate occlusion and deflation of the cuffs during intervals of 5-6 heart beats, maximal blood flow is determined during the hyperemic response immediately following exercise. Measurements of the flow during exercise cannot be performed simply with venous occlusion plethysmography because the muscles move strongly and the volume changes during the contractions. Measurements of post-exercise hyperaemia, on the other hand, can be performed technically simply. Calf or forearm blood flow can be calculated from the rate of the initial increase in calf or arm circumference during venous occlusion and is expressed as milliliters per 100 ml of calf tissue per minute (52, 53, 54). Equipment is available for bilateral measurements at the Vascular laboratory of the Department of Medicine.

I.1.3. Skin flow assessment using laser Doppler flowmetry.

Laser Doppler flowmetry is a technique for measuring changes in tissue blood flow. The method is based on the Doppler shift of monochromatic laser light scattered back by tissue. The Doppler signal is determined by the velocity and the number of moving scattering particles, mainly red blood cells and therefore is directly related to tissue blood flow rate. This non-invasive method is applicable to almost every tissue, although it has until now mainly been used for measuring skin blood flow. In the present study we will use a Diodop LDF meter, connected to a PC with self-developed software for signal analysis. Measurements will be made at rest after 3 minute of suprasystolic occlusion. A biological zero will be obtained. Local skin temperature will also be recorded using an Ellab thermocouple thermometer. Measurements will be performed at the medial malleolus of the treated leg, in case open lesions are present another site at the foot will be used (55, 56, 57, 58).

Optional (if possible combined with fluorescein angiography of the eyes):

I.1.4. Fluoresceine capillary microscopy.

In the skin the transcapillary diffusion of intravenously injected fluorescein is used to measure vascular permeability. Using single capillary fluorescein videodensitometry, Bollinger et al. have demonstrated that fluorescein diffusion in the skin is increased in a group of subjects with long-term diabetes mellitus (Bollinger 1982). This observation was confirmed by the same group, using a "large window" method (which is comparable to the method we used in our study), in which a larger number of capillaries, up to approximately 100, is studied. The large window method is, therefore, less sensitive for spatial and temporal changes in capillary flow. We have considerably improved the reproducibility of this method and thereby its value as a tool in intervention studies, without sacrificing its power to discriminate diabetic from normal subjects. Use is made of custom-made equipment, described elsewhere in detail (59, 60, 61, 62).

I.1.5. Positron emission tomography (PET).

Local perfusion and reduction of ischemic areas are to be detected by PET; H_2O^{15} -PET (perfusion) or F-deoxyglucose (metabolism).

I.2. Ophthalmic examination.

The following ophthalmic examinations will be conducted and recorded:

Ophthalmic history, best corrected visual activity, slit-lamp biomicroscopy, intraocular pressure, fundus examination, fundusphotography and fluorescein angiography. Patients are categorized according to their clinical examination in having no retinopathy, background, preproliferative and proliferative diabetic retinopathy, based on the ETDRS international classification.

Seven standard fundus photographs of both eyes are made through dilated pupils.

Fluorescein angiography is performed of the macular area of the right eye and of the mid periphery of both eyes in the late phases (64, 65, 66).

I.3. Neurologic examination.

Neurological examination, the same measurements as performed at pretreatment assessment.

J. Follow-up assessments:

- * Physical examinations.
- * Vascular techniques: angiography, ankle-, (optional) thigh-, and toe pressures and -brachial index, laser Doppler flowmetry. Before and at the end of follow-up.
- * Ophthalmological examination; funduscopy and fundus photographs.
- * Neurological examination.
- * SMAC-20.

K. Potential side effects.

A. Complications related to diagnostic angiography such as rupture of an artery, infection, embolization, or allergic reactions to the contrast media used.

B. Even though we have attempted to minimize the risks associated with gene transfer by eliminating the need for any viral, liposomal or other vectors, it is recognized that theoretical risks of gene transfer remain. Though the DNA to be transferred is considered to be harmless, events could occur within normal cells that take up the foreign DNA that allow them to be transformed.

Laboratory studies suggest that this is unlikely. We nevertheless acknowledge that cells could theoretically become abnormal after long periods of time.

C. Complications related to the VEGF protein. Until now only oedema of the treated limb has been described as minor complication. No evidence for systemic complications such as induction / exacerbation.

L. Definition of response

Response will be defined as limb survival and/or improvement in ABI of 15%, as two independent variables. Limb survival is defined as absence of major amputation. Major amputations are those at the level of the ankle or higher.

M. Risk-Benefit Analysis

In the patient the major benefit we anticipate is to prevent major amputation. The risk can be toxicity as nephropathy, retinopathy and oedema and secondary infections due to the local injections.

On the basis of pre-clinical animal studies, we do not anticipate adverse consequences.

In the absence of gene transfer, deleterious consequences might include persistent rest pain, deterioration of the limb ischemia.

N. Evaluation of safety

Following intramuscular injection toxicity will be evaluated to WHO criteria. No further injections will be given in case of any grade 3 or 4 toxicity which is caused by the intramuscular injections.

O. Statistics

The natural history for the need for major amputations of non reconstructable CLI (with ankle systolic pressure < 50 mm Hg) is approximately 50% in 100 days. Based on the results of Isner demonstrating an approximately 50% salvage rate in limbs destined to be amputated when VEGF started, we hope for a substantial improvement in amputation rate. As treatment starts early we

estimate that at least 50% of limbs at risk will not be deteriorate to the point of major amputation. From these patients who deteriorate a 50% salvage rate might still be feasible. Therefore, an improvement of approximately 50% major amputation to 12.5% is expected in this study. To detect the expected 37.5% improvement of limb survival (50% amputations to 12.5%) or a 50% improvement of ABI of 15% with the treatment of VEGF compared to the control group, 26 patients are needed in both groups (significance level of 0.05 (one-sided) and power 0.85; according to a binomial distribution). In total 60 patients will be entered because of a drop out percentage of appr. 10% in these group of patients. There will be a block randomization for two times 30 patients. After treating 30 patients there will be an interim analysis. The study will be stopped if there is a significant difference between both groups after 30 patients have been entered.

P. Subject discontinuation

Subjects may discontinue the study at any time. If, in the judgment of the investigators, continuation in the study would be detrimental to the subject, the investigator may withdraw the subject at any time.

If a subject withdraws from the study after 24 hours, but before the safety and biologic activity follow-up period, he or she should be contacted in order to obtain information about the reason(s) for his or her withdrawal and the occurrence of any adverse events. The subject should be urged to return to the clinic for an early discontinuation visit to be assessed for safety and biologic response.

Q. Randomisation

This is a double blind placebo controlled phase III study. The placebo is produced in the pharmacy. There will be a block randomization for two times 30 patients and an interim analysis. Randomization will be done by sealed envelope method without replacement.

R. Results:

Preliminary results show that administration of VEGF gene to patients with critical limb ischemia has a distinct positive effect as compared tot the control group.

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Claims

1. A vector which is capable of effecting the expression of a vascular
5 endothelial growth factor (VEGF or a functional equivalent thereof) in an
appropriate environment wherein said vector comprises a modified pCMV
promoter.
2. A vector according to claim 1 wherein said modification comprises the
insertion of a 9bp nucleic acid sequence ACGCGCGCT, wherein A is a
10 deoxyadenyl, G is deoxyguanyl, C is deoxycytosyl and T is thymidyl.
3. A vector according to claim 1 or 2 wherein said vector comprises an
antibiotic resistance gene.
4. A vector according to claim 3 wherein said antibiotic resistance gene
comprises kanamycin.
- 15 5. A vector according to anyone of claims 1-4 wherein said vector comprises a
5' HSV translation initiation signal and/or a β -globin splicing/adenylation
signal.
6. A vector according to anyone of claims 1-5 wherein said vector comprises a
ColE1-like replicon and/or an F1 replication origin.
- 20 7. A vector according to anyone of claims 1-6 wherein said vascular endothelial
growth factor (VEGF) is derived from a mammal.
8. A vector according to claim 7 wherein said mammal is a human.
9. A vector according to anyone of claims 1-8 wherein said vector comprises a
vascular endothelial growth factor (VEGF) nucleic acid.
- 25 10. A vector according to anyone of claims 1-9 wherein said nucleic acid is
operative in mammalian systems.
11. A vector according to claim 10 wherein said vector can replicate to a high
copy number in a bacterial cell.
12. A host cell which comprises a vector according to anyone of claims 1-11.
- 30 13. A host cell according to claim 12 which is human.

14. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament for the regulation of endothelial function.
15. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament for the regulation of angiogenesis.
- 5 16. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament for the regulation of arteriogenesis.
17. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament to induce the release of hematopoietic growth factors by bone marrow endothelial cells.
- 10 18. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament to induce the development of supplemental collateral blood vessels.
19. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament to induce therapeutic angiogenesis
- 15 20. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament to induce transendothelial progenitor cell migration.
21. Use of a vector according to anyone of claims 1-11 in the preparation of a medicament to increase endothelial fenestration.
22. A composition comprising a vector according to anyone of claims 1-11.
- 20 23. A composition according to claim 22 which is a pharmaceutical composition.
24. Use of a composition according to claim 22 or 23 and/or a vector according to anyone of claim 1-11 for the treatment of arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders, in particular tissue ischemia especially associated with diabetes.
- 25 25. A method of treatment of arterial diseases and/or bone marrow diseases and/or neural diseases and/or inflammatory disorders, comprising administering a vector according to anyone of claims 1-11 and/or a composition according to claim 22 or 23 with a carrier to a suitable
- 30 recipient.

1/2

Figure 1. Plasmid pVEGF165.MB sequence

Sequence:

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1 GAATTCGAGC TCGCCCCGTT ACATAACTTA CGGTAAATGG CCCGCCTGGC
51 TGACCGCCCCA ACGACCCCCG CCCATTGACG TCAATAATGA CGTATGTTCC
101 CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT
151 TACGGTAAAC TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT
201 ACGCCCCCTA TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC
251 CCAGTACATG ACCTTATGGG ACTTTCCTAC TTGGCAGTAC ATCTACGCGC
301 GCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC
351 ATCAATGGGC GTGGATAGCG GTTTGACTCA CGGGGATTTT CAAGTCTCCA
401 CCCCATTGAC GTCAATGGGA GTTTGTTTGG GCACCAAAAT CAACGGGACT
451 TTCCAAAATG TCGTAACAAC TCCGCCCCAT TGACGCAAAT GGGCGGTAGG
501 CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCGTTTAG TGAACCGTCA
551 GATCGCCTGG AGACGCCATC CACGCTGTTT TGACCTCCAT AGAAGACACC
601 GGGACCGATC CAGCCTCCGG GGGATCTTGG TGGCGTGAAA CTCCCGCACC
651 TCTTCGGCCA GCGCCTTGTA GAAGCGCGTA CGATGAACTT TCTGCTGTCT
701 TGGGTGCATT GGAGCCTTGC CTTGCTGCTC TACCTCCACC ATGCCAAGTG
751 GTCCCAGGCT GCACCCATGG CAGAAGGAGG AGGGCAGAAT CATCACGAAG
801 TGGTGAAGTT CATGGATGTC TATCAGCGCA GCTACTGCCA TCCAATCGAG
851 ACCCTGGTGG ACATCTTCCA GGAGTACCCT GATGAGATCG AGTACATCTT
901 CAAGCCATCC TGTGTGCCCC TGATGCGATG CGGGGGCTGC TGCAATGACG
951 AGGGCCTGGA GTGTGTGCCC ACTGAGGAGT CCAACATCAC CATGCAGATT
1001 ATGCGGATCA AACCTCACCA AGGCCAGCAC ATAGGAGAGA TGAGCTTCCT
1051 ACAGCACAAC AAATGTGAAT GCAGACCAAA GAAAGATAGA GCAAGACAAG
1101 AAAATCCCTG TGGCCCTTGC TCAGAGCGGA GAAAGCATTT GTTTGTACAA
1151 GATCCGAGCA CGTGTAATG TTCCTGCAAA AACACAGACT CGCGTTGCAA
1201 GCGGAGGCAG CTTGAGTTAA ACGAACGTAC TTGCAGATGT GACAAGCCGA
1251 GCGGGTGAGG ATCCTGAGAA CTTCAGGGTG AGTTTGGGGA CCCTTGATTG
1301 TTCTTTCTTT TTCGCTATTG TAAATTCAT GTTATATGGA GGGGGCAAAG
1351 TTTTCAGGGT GTTGTTTAGA ATGGGAAGAT GTCCCTTGTA TCACCATGGA
1401 CCCTCATGAT AATTTTGTTC CTTTCACTTT CTA CTCTGTTT GACAACCATT
1451 GTCTCCTCTT ATTTTCTTTT CATTTTCTGT AACTTTTTTCG TTAACCTTTA
1501 GCTTGCATTT GTAAAGCAAT TTTAAATTC CTTTTGTGTTA TTTGTCAGAT
1551 TGTAAGTACT TTCTCTAATC ACTTTTTTTT CAAGGCAATC AGGGTATATT
1601 ATATTGTACT TCAGCAGAT TTTAGAGAAC AATTGTTATA ATTAAATGAT
1651 AAGGTAGAAT ATTTCTGCAT ATAAATTC TGCTGGCGTG AAATATTCTT
1701 ATTGGTAGAA ACAACTACAC CCTGGTCATC ATCCTGCCTT TCTCTTTATG
1751 GTTACAATGA TATACACTGT TTGAGATGAG GATAAAATAC TCTGAGTCCA
1801 AACCGGGGCC CTCTGCTAAC CATGTTTCATG CCTTCTTCTC TTTCCTACAG
1851 CTCCTGGGCA ACGTCTGGT TGTGTGCTG TCTCATCAT TTGGCAAAGA
1901 ATTCACTCCT CAGGTGCAGG CTGCCTATCA GAAGGTGTG TGCTGTGTGG
1951 CCAATGCCCT GGCTCACAAA TACCACTGAG ATCTTTTTTCC CTCTGCCAAA
2001 AATTATGGGG ACATCATGAA GCCCCTTGAG CATCTGACTT CTGGCTAATA
2051 AAGGAAATTT ATTTTCATTG CAATAGTGTG TTGGAATTTT TTGTGTCTCT
2101 CACTCGGAAG GACATATGGG AGGGCAAATC ATTTAAAACA TCAGAATGAG
2151 TATTTGGTTT AGAGTTTGGC AACATATGCC CATATGCTGG CTGCCATGAA
2201 CAAAGGTTGG CTATAAGAG GTCATCAGTA TATGAAACAG CCCCCTGCTG
2251 TCCATTCCCTT ATTCCATAGA AAAGCCTTGA CTTGAGGTTA GATTTTTTTT
2301 ATATTTTGT TGTGTTTATT TTTTCTTTA ACATCCCTAA AATTTTCCTT
2351 ACATGTTTTA CTAGCCAGAT TTTTCTCTCT CTCCTGACTA CTCCCAGTCA
2401 TAGCTGTCCC TCTTCTCTTA TGGAGATCCC TCGAGGAGCT TTTTGC AAAA
2451 GCCTAGGTAA TGAATCGGCC AACGCGCGGG GAGAGGCGGT TTGCGTATTG
2501 GCGGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC GGTCGTTCGG
2551 CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC
2601 AGAATCAGGG GATAACGAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA
2651 AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC
2701 CGCCCCCTTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG
2751 AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCTCT GGAAGCTCCC
2801 TCGTGGGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA CCTGTCCGCC
2851 TTTCTCCCTT CGGGAAGCGT GCGGCTTTCT CATAGCTCAC GCTGTAGGTA
2901 TCTCAGTTCT GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT GTGCACGAAC
2951 CCCCCGTTCA GCCCCACCGC TGCGCCTTAT CCGGTAAC TA TCGTCTTGAG
3001 TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG CCACTGGTAA

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2/2

Figure 1, Contd.

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3051 CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT
3101 GGTGGCCTAA CTACGGCTAC ACTAGAAGAA CAGTATTTGG TATCTGCGCT
3151 CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG
3201 CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTCG AAGCAGCAGA
3251 TTACGCGCAG AAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG
3301 GGGTCTGACG CTCAGTGGAA CGAAACTCA CGTTAAGGGA TTTTGGTCAT
3351 GAGATTATCA AAAAGGATCT TCACCTAGAC TGCCAGTCAA AGCCACGTTG
3401 TGTCTCAAAA TCTCTGATGT TACATTGCAC AAGATAAAAA TATATCATCA
3451 TGAACAATAA AACTGTCTGC TTACATAAAC AGTAATACAA GGGGTGTTAT
3501 GAGCCATATT CAACGGGAAA CGTCTTGCTC GAGGCCGCGA TTAAATTCCA
3551 ACATGGATGC TGATTTATAT GGGTATAAAT GGGCTCGCGA TAATGTCGGG
3601 CAATCAGGTG CGACAATCTA TCGATTGTAT GGGAAGCCCG ATGCGCCAGA
3651 GTTGTTCCTG AAACATGGCA AAGGTAGCGT TGCCAATGAT GTTACAGATG
3701 AGATGGTCAG ACTAACTGG CTGACGGAAT TTATGCCTCT TCCGACCATC
3751 AAGCATTTTA TCCGTACTCC TGATGATGCA TGGTTACTCA CCACTGCGAT
3801 CCCCAGGAAA ACAGCATTCC AGGTATTAGA AGAATATCCT GATTCAGGTG
3851 AAAATATTGT TGATGCGCTG GCAGTGTTCG TGCGCCGGTT GCATTCGATT
3901 CCTGTTTGTA ATTTCTCTTT TAACAGCGAT CGCGTATTTT GTCTCGCTCA
3951 GCGCAATCA CGAATGAATA ACGGTTTGGT TGATGCGAGT GATTTTGATG
4001 ACGAGCGTAA TGGTGGCCT GTTGAACAAG TCTGGAAAGA AATGCATAAG
4051 CTTTGGCCAT TCTACCGGA TTCAGTCGTC ACTCATGGTG ATTTCTCACT
4101 TGATAACCTT ATTTTTCAGC AGGGGAAAT AATAGGTTGT ATTGATGTTG
4151 GACGAGTCGG AATCGCAGAC CGATACCAGG ATCTTGCCAT CCTATGGAAC
4201 TGCCCTCGGTG AGTTTTCTCC TTCATTACAG AAACGGCTTT TTCAAAAATA
4251 TGGTATTGAT AATCCTGATA TGAATAAATT GCAGTTTCAT TTGATGCTCG
4301 ATGAGTTTTT CTAATCAGAA TTGGTTAATT GGTGTGAACA CTGGCAGAGC
4351 ATTACGCTGA CTTGACGGGA CGGCGGCTTT GTTGAATAAA TCGAACTTTT
4401 GCTGAGTTGA AGGTACAGAT CACGCATCTT CCCGACAACG CAGACCGTTC
4451 CGTGGCAAAG CAAAAGTTCA AAATCACCAA CTGGTCCACC TACAACAAAG
4501 CTCTCATCAA CCGTGGCTCC CTCACCTTCT GGCTGGATGA TGGGGCGATT
4551 CAGGCCTGGT ATGAGTCAGC AACACCTTCT TCACGAGGCA GACCTCAGCG
4601 CCCCCCCCCC CCGTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG
4651 TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC
4701 ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA
4751 ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA
4801 CTCTTCTCTT TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT
4851 GAGCGGATAC ATATTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTT
4901 CGCGCACATT TCCCCGAAA GTGCCACCTG ACGTCTAAGA AACCATTATT
4951 ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTCT
5001 CGCGCGTTTC GGTGATGACG GTGAAAACCT CTGACACATG CAGCTCCCGG
5051 AGACGGTCAC AGCTGTCTG TAAGCGGATG CCGGGAGCAG ACAAGCCCGT
5101 CAGGGCGCGT CAGCGGTGT TGGCGGGTGT CGGGGCTGGC TTAACATATG
5151 GGCATCAGAG CAGATTGTAC TGAGAGTGCA CCATATGCGG TGTGAAATAC
5201 CGCACAGATG CGTAAGGAGA AAATACCGCA TCAGGAAATT GTAAGCGTTA
5251 ATATTTTGTT AAAATTTCGG TTAAATTTT GTTAAATCAG CTCATTTTTT
5301 AACCAATAGG CCGAAATCGG CAAAATCCCT TATAAATCAA AAGAATAGAC
5351 CGAGATAGGG TTGAGTGTG TTCCAGTTT GAACAAGAGT CCACATTTAA
5401 AGAACGTGGA CTCCAACGTC AAAGGGCGAA AAACCGTCTA TCAGGGCGAT
5451 GGCCCACTAC GTGAACCATC ACCCTAATCA AGTTTTTTGG GGTGAGGTG
5501 CCGTAAAGCA CTAATTCGGA ACCCTAAAGG GAGCCCCCGA TTTAGAGCTT
5551 GACGGGGAAA GCCGGCGAAG GTGGCGAGAA AGGAAGGGAA GAAAGCGAAA
5601 GGAGCGGGCG CTAGGCGGCT GGCAAGTGTA GCGGTCACGC TGCGCGTAAC
5651 CACCACACCC GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TCCATTCGCC
5701 ATTGAGGCTG CGCAACTGTT GGAAGGGCG ATCGGTGCGG GCCTCTTCGC
5751 TATTACGCCA GCTGCGGAAA GGGGGATGTG CTGCAAGGCG ATTAAGTTGG
5801 GTAACGCCAG GGTTCCTCCA GTCACGACGT TGTAAACGA CGGCCAGTGA
5851 ATTGTAATAC GACTCACTAT AGGGC

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **VECTORS FOR ENHANCED EXPRESSION OF VEGF FOR DISEASE TREATMENT**

(57) Abstract: The invention provides a vector which is capable of the expression of a vascular endothelial growth factor wherein said vector comprises a modified pCMV promoter. The invention further provides use of a vector which is capable of and expressing a vascular endothelial growth factor (VEGF) for the regulation of endothelial function, angiogenesis and arteriogenesis. The invention further comprises use of a vector which is capable of the expression of a vascular endothelial growth factor (VEGF) for the prophylactic treatment of arterial diseases and/or bone marrow diseases and/or neural diseases.



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INTERNATIONAL SEARCH REPORT

Intern: Application No
PCT/NL 02/00326

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K48/00 A61K121/00 C12N15/67

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBL, BIOSIS, WPI Data, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| X | WO 99 47690 A (INTROGEN THERAPEUTICS INC ;CHADA SUNIL (US); ALMOND BRIAN D (US);) 23 September 1999 (1999-09-23) the whole document | 1 |
| X | WO 00 61751 A (CHANG BEY DIH ;UNIV ILLINOIS (US); RONINSON IGOR B (US)) 19 October 2000 (2000-10-19) the whole document | 1 |
| X | US 5 919 652 A (BELLDEGRUN ARIE S ET AL) 6 July 1999 (1999-07-06) the whole document | 1 |
| | --- -/- | |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

27 March 2003

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Petri, B

INTERNATIONAL SEARCH REPORT

Inter Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | DATABASE EMBL 'Online! retrieved from EBI Database accession no. AF264696 XP002180574 | 1 |
| Y | the whole document | 2-23 |
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 02/00326

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 22-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter Application No

PC 1 / NL 02/00326

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